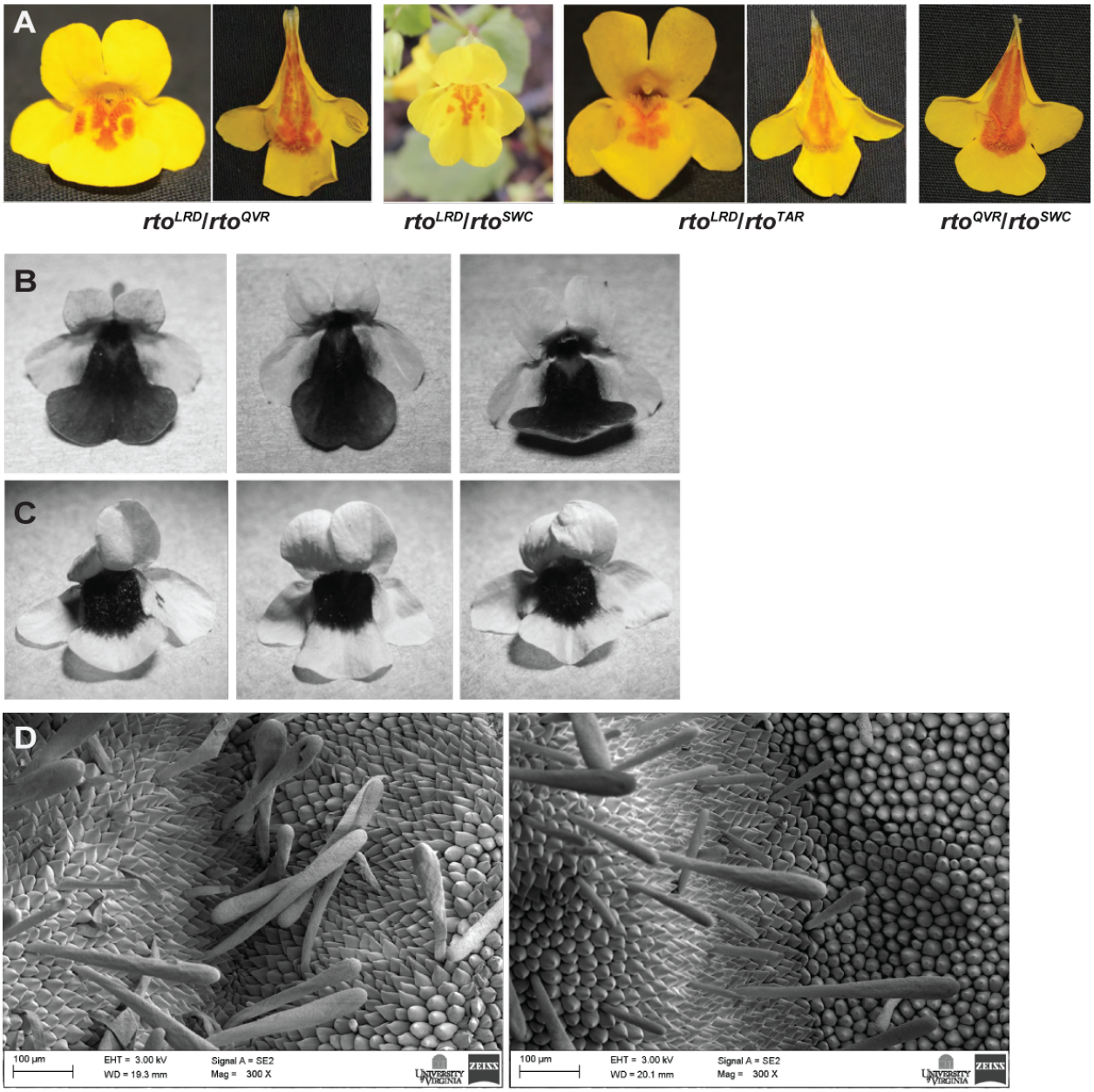

Figure S1. Additional phenotypic characterization of *Mimulus guttatus* genotypes. Related to Figure 1 and Table 1. (A) Complementation crosses among the natural *rto*-like variants of *M. guttatus*. These crosses suggest that all four natural variants are different alleles of the same locus. (B) UV spectrum images of full-sib RTO^{SWC}/RTO^{SWC} (left), RTO^{SWC}/rto^{SWC} (center), and rto^{SWC}/rto^{SWC} (right) flowers. (C) UV spectrum images of RTO^{LRD}/RTO^{LRD} (left), RTO^{LRD}/rto^{LRD} (center), and rto^{LRD}/rto^{LRD} (right) flowers. (D) SEM of ventral petal conical cells and trichomes in full-sib RTO^{SWC}/RTO^{SWC} (left) and rto^{SWC}/rto^{SWC} (right) flowers.

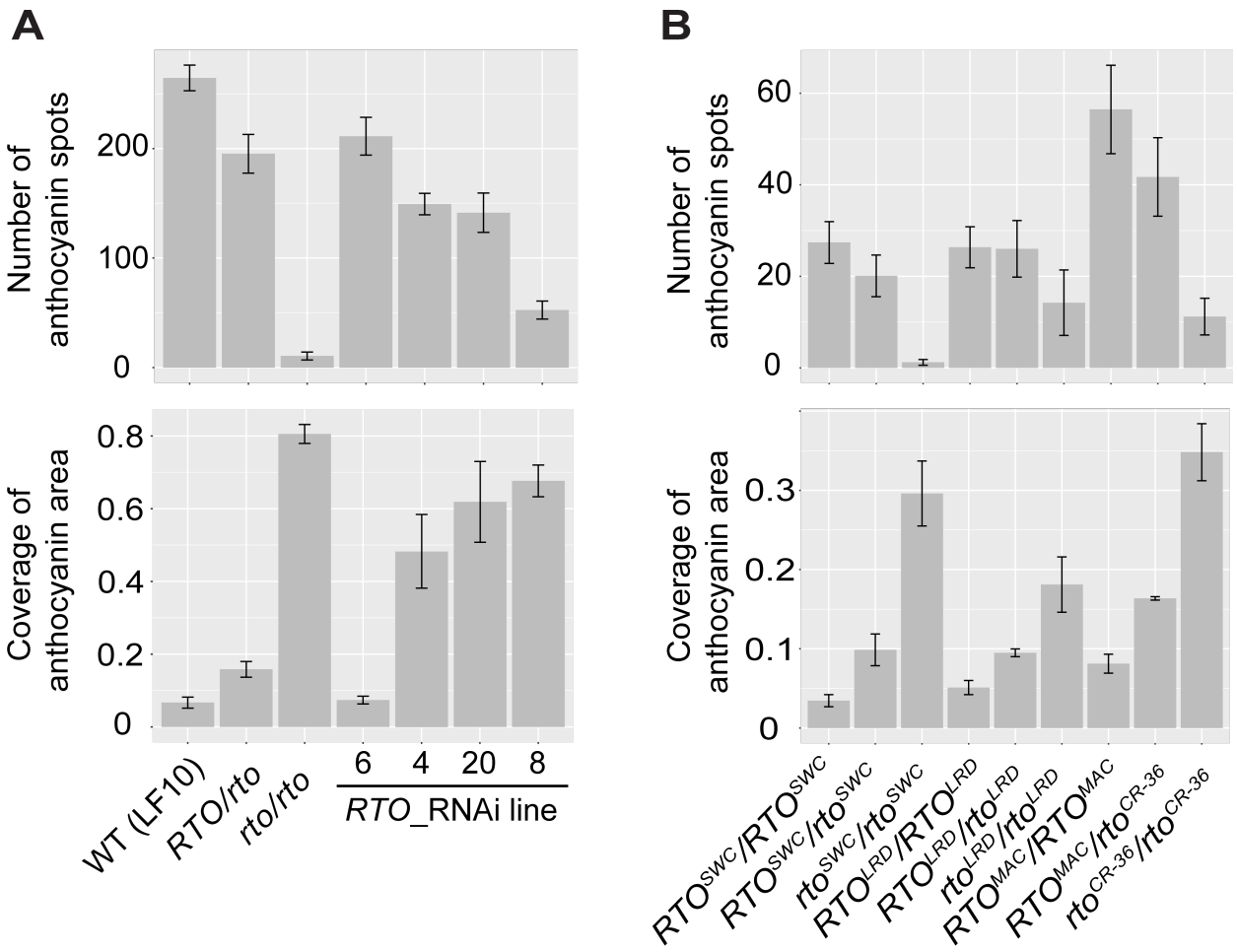

Figure S2. Quantification of the anthocyanin phenotypes in the nectar guides of *Mimulus lewisii* and *M. guttatus*. Related to Figures 1, 3, and 4. Note that the coverage of anthocyanin area was calculated using different total areas for *M. lewisii* (A) and *M. guttatus* (B), and therefore the metric is not directly comparable between the two species. For *M. lewisii*, the total area is the yellow part of the ventral petal (i.e., the nectar guides). For *M. guttatus*, because the entire ventral petal is yellow and there is no clear boundary between the nectar guides and the rest of the petal, the entire ventral petal was used as the total area.

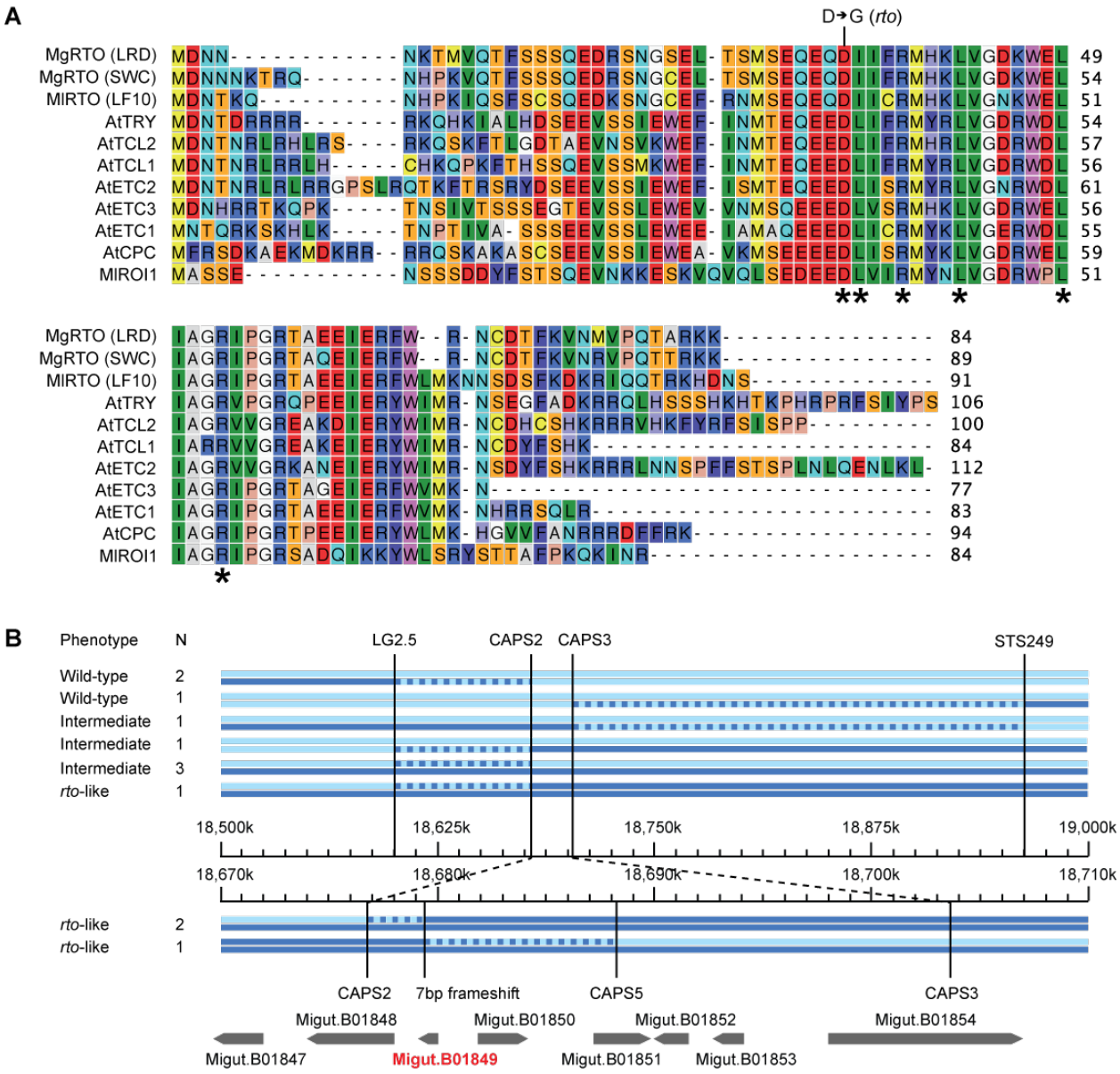

Figure S3. *RTO* sequence characteristics and fine mapping. Related to Figure 2. (A) Alignment of the R3-MYB amino acid sequences of *Mimulus* and their homologues in *Arabidopsis*. The bHLH-interacting motif ([DE]Lx$_2$[RK]x$_3$Lx$_6$Lx$_3$R) is marked by the asterisks. The D->G amino acid replacement in the *M. lewisii rto* allele is highlighted above the alignment. (B) Fine mapping of the *rto*^{SWC} allele along the relevant section of *M. guttatus* pseudochromosome 2. The upper chromosome representations reflect allelic identities along the full chromosomal interval, and the lower chromosome representations reflect allelic identities just within the sub-interval between markers CAPS2 and CAPS3. Light and dark blue segments represent wild-type and mutant parent haplotypes, respectively. Dashed segments indicate a recombination event present somewhere along the segment. The phenotype and N columns indicate the phenotype and number of individuals, respectively.

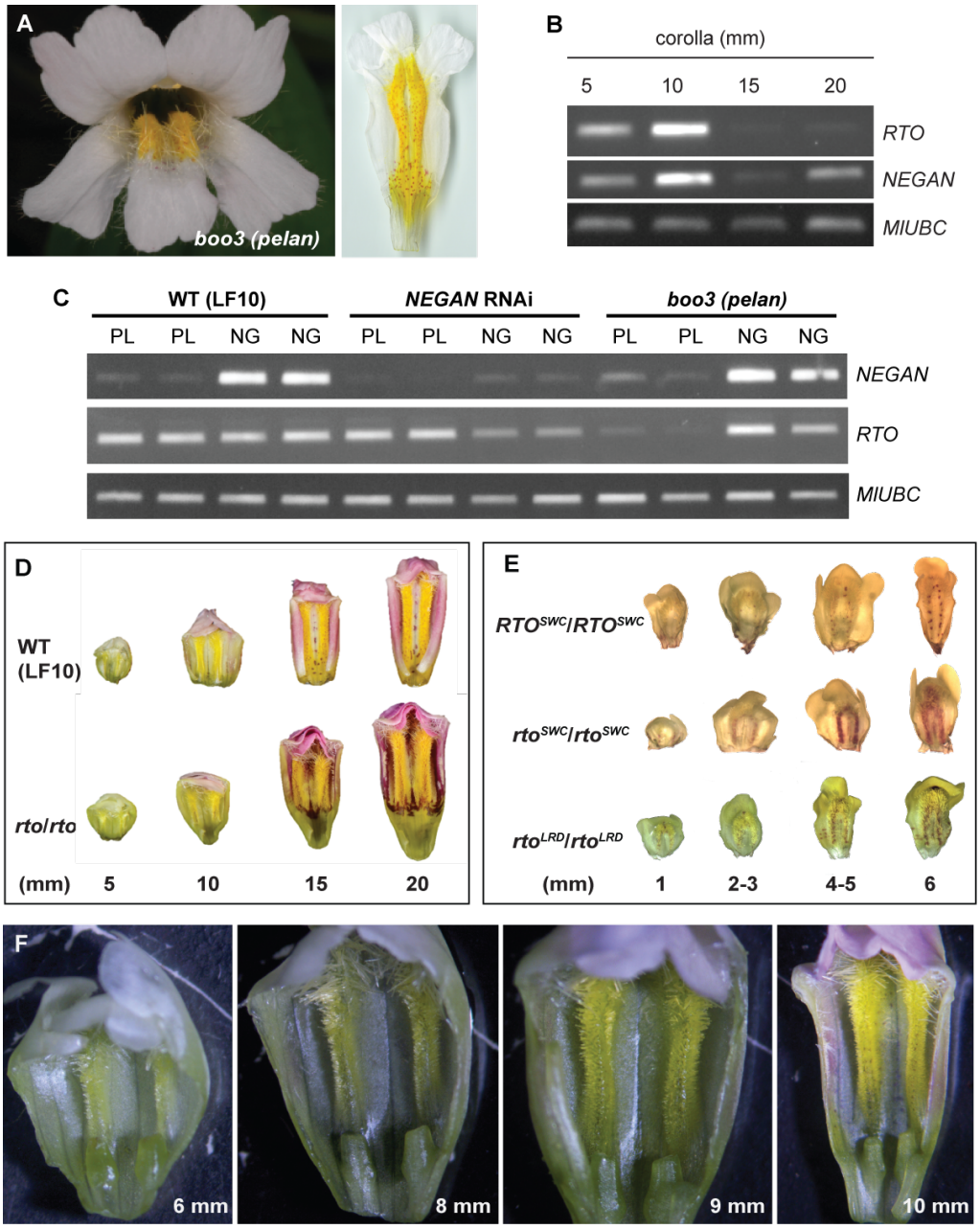

Figure S4. *RTO* and *NEGAN* expression and flower developmental stages. Related to Figures 2 and 3. (A) Flower phenotype of the *Mimulus lewisii pelan* mutant. (B) Both *RTO* and *NEGAN* show peak expression level at 10-mm flower bud developmental stage in *M. lewisii*. (C) Relative expression of *NEGAN* and *RTO* in *M. lewisii* petal lobes (PL) vs. nectar guides (NG) at 10-mm flower bud stage. In the wild-type (WT), *NEGAN* is preferentially expressed in the nectar guides, whereas *RTO* is expressed in both the petal lobes and the nectar guides. *RTO* expression is down-regulated in the nectar guides but is unaffected in the petal lobes in the *NEGAN* RNAi line. Conversely, *RTO* expression is unaffected in the nectar guides but down-regulated in the petal lobes in the *pelan* mutant. Two biological replicates were used for each tissue and genotype. *MlUBC* was used as the reference gene. (D and E) Corresponding developmental stages of anthocyanin spot formation in *M. lewisii* (D) and *M. guttatus* (E). (F) Detailed view of the *M. lewisii* nectar guides between 6-mm and 10-mm stages. Anthocyanin spots are not visible in the nectar guides even at 9-mm stage but become visible at 10-mm stage.

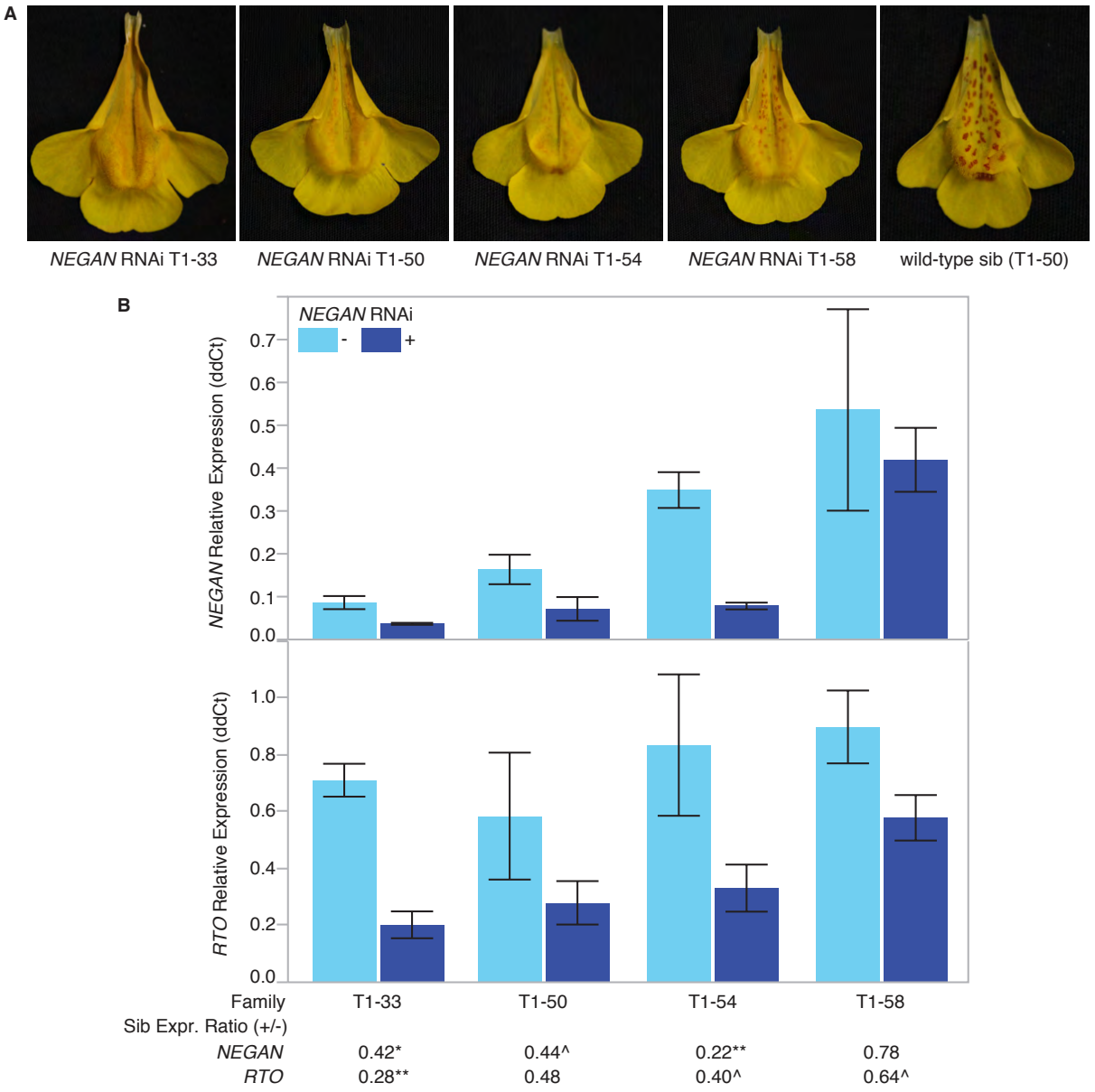

Figure S5. RNAi knockdown of *MgNEGAN* in *M. guttatus*. Related to Figure 3. (A) Ventral and lateral petals from four independent T_1 families carrying a *NEGAN* RNAi transgene and exhibiting a range of phenotypes, from complete to weak reduction of anthocyanin spot formation. The rightmost image is from a full-sib T_1 plant that did not inherit the *NEGAN* RNAi transgene from its T_0 parent and shows the wild-type phenotype for comparison. (B) Expression levels of *NEGAN* and *RTO* in the four T_1 families depicted in (A), as measured by qRT-PCR. Relative expression is reported as the mean ddCt ± s.e. for three biological replicates per genotype per line, normalized to the highest value across the experiment. The value used for each individual biological replicate is the mean of three technical replicates. "+" (dark blue) and "-" (light blue) indicate full sibs in each family that do and do not carry the *NEGAN* RNAi transgene, respectively. The ratio of the "+" mean to the "-" for each T_1 family is also shown. Significant differences in expression between "+" and "-" sibs were tested by a one-tailed t-test (^$P < 0.1$, *$P < 0.05$, **$P<0.01$).

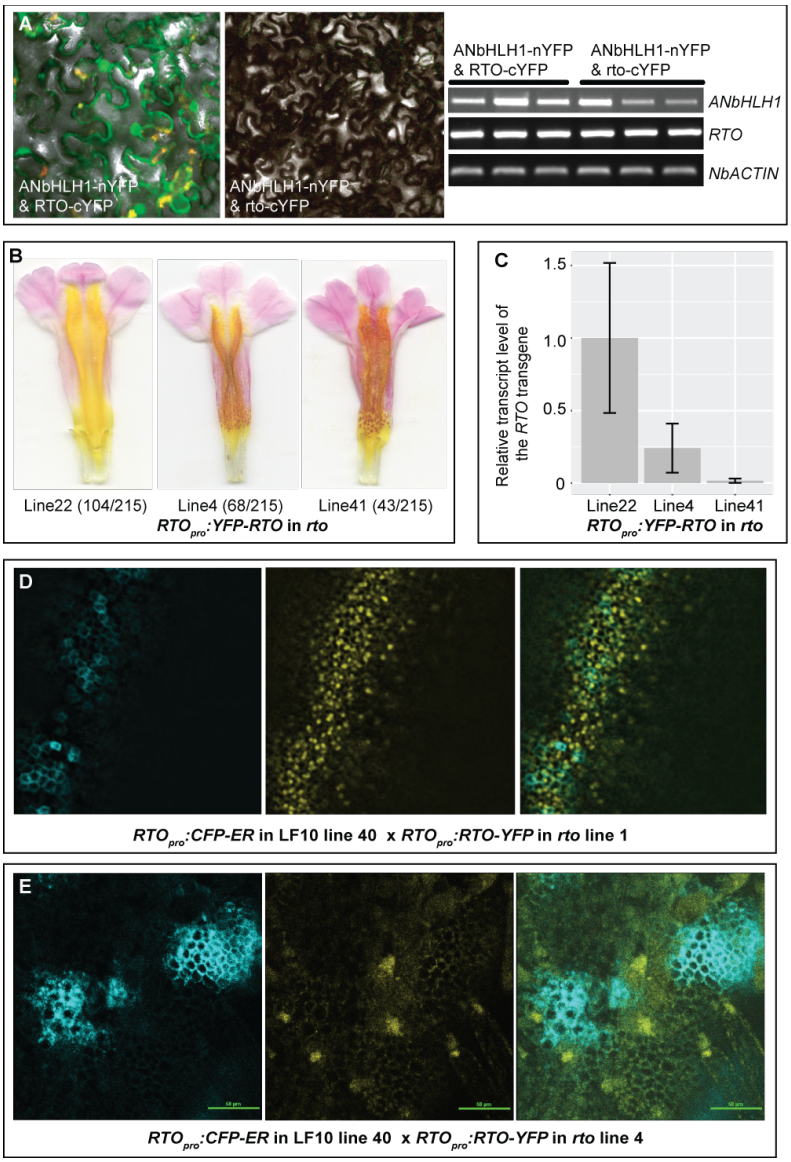

Figure S6. Additional functional characterization of *RTO*. Related to Figures 3 and 5. (A) Additional BiFC images showing that the wild-type RTO protein interacts with ANbHLH1, whereas the mutant rto protein does not. Relative expression levels of *ANbHLH1* and *RTO* were assayed with semi-quantitative RT-PCR four days after agroinfiltration. Three biological replicates are shown. The *Nicotiana benthamiana ACTIN* gene was used as the reference gene. (B) Dissected flower images of RTO_{pro}*:YFP-RTO* transgenic lines in the *Mimulus lewisii rto* background, showing the anthocyanin patterns in the nectar guides. Numbers of over-rescued, partially rescued, and non-rescued transgenic lines are indicated in the parentheses under the corresponding phenotypes. (C) The relative transcript level of the transgene, as measured by qRT-PCR and normalized to the highest value across the experiment, is correlated with the phenotype. *MlUBC* was used as the reference gene. Error bars represent 1 SD from three biological replicates. (D and E) Additional independent crosses that generated transgenic plants with both RTO_{pro}*:CFP-ER* and RTO_{pro}*:YFP-RTO*, showing a broader distribution of RTO protein (yellow) than *RTO* promoter activity (blue). CFP and YFP signals were imaged with the excitation wavelength of 457 nm and 514 nm, respectively. The right image is an overlay between the left and the center.

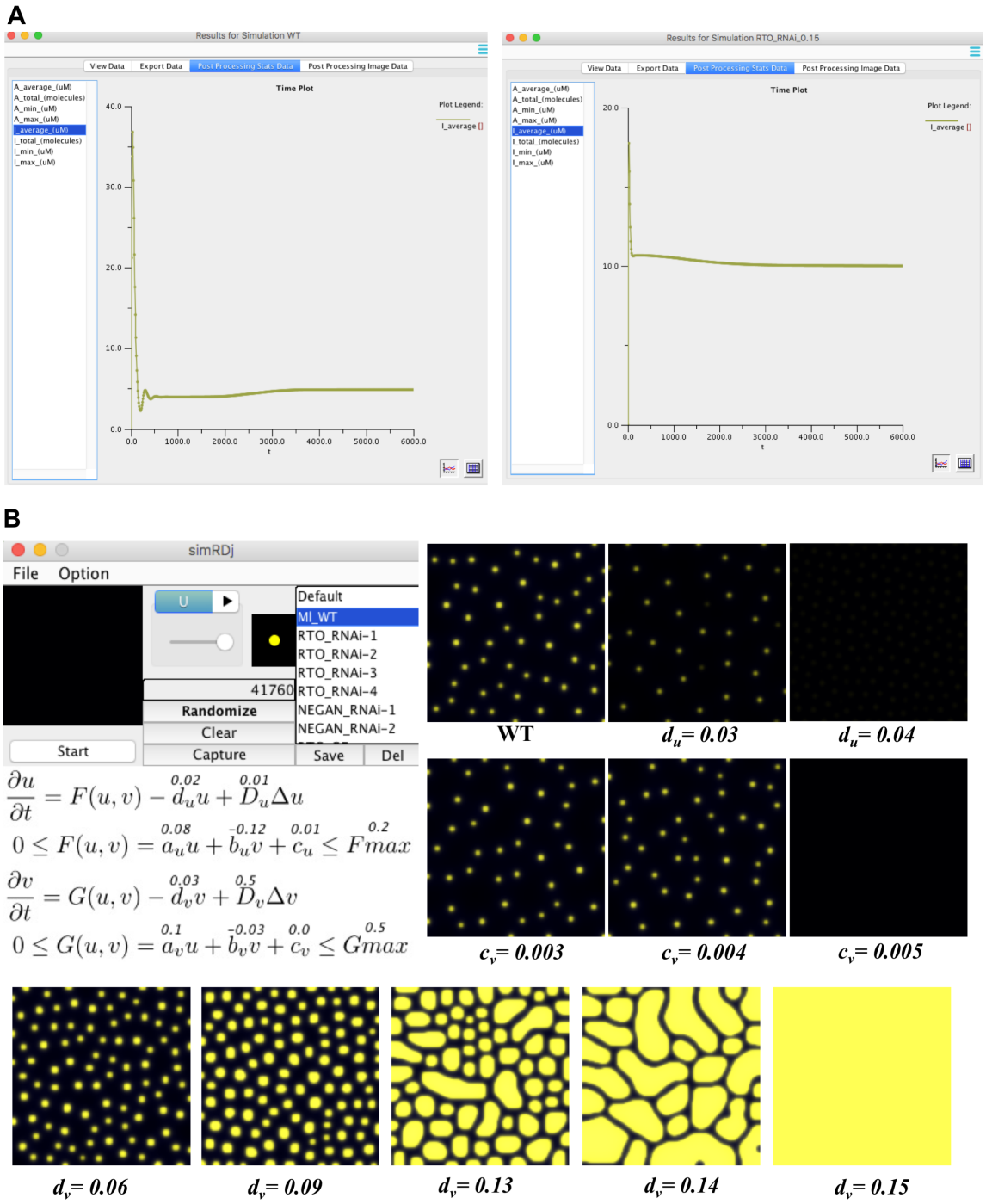

Figure S7. Computer simulation of the anthocyanin spot patterning. Related to Figure 6. (A) Screenshots of Post Processing Stats Data of the VCell simulations, showing average inhibitor levels of the wild-type (WT) condition (left; ~5.0) and one of the *RTO* RNAi conditions (right; ~10.0). Simulations under other *RTO* RNAi conditions show similar results, which are publicly available in the VCell software (http://vcell.org). (B) Computer simulations with the RD model implemented in SimRDj generated similar results as the VCell simulations. Shown on the upper left is a screenshot of the SimRDj platform, showing the partial differential equations and the parameter values used for simulating the WT pattern. All other patterns were simulated with the same parameter values as in the WT, except one modification for each perturbation as shown below each panel.

Trial	Genotype	No. Open Flowers	Observed Visits	Expected Visits	G-value	*P*
1	*RTO/RTO*	69	21	16.62	1.02	0.31
	rto/rto	68	12	16.38		
2	*RTO/RTO*	9	15	25.20	4.37	0.04
	rto/rto	6	27	16.80		
3	*RTO/RTO*	5	24	27.27	0.32	0.57
	rto/rto	6	36	32.73		
4	*RTO/RTO*	7	37	31.50	1.03	0.31
	rto/rto	5	17	22.50		
5	*RTO/RTO*	6	23	26.18	0.37	0.54
	rto/rto	5	25	21.82		
6	*RTO/RTO*	17	46	43.07	0.14	0.71
	rto/rto	28	68	70.93		
7	*RTO/RTO*	4	21	21.14	0.00	0.98
	rto/rto	3	16	15.86		
8	*RTO/RTO*	23	26	28.06	0.12	0.73
	rto/rto	27	35	32.94		
9	*RTO/RTO*	15	16	32.79	6.45	0.01
	rto/rto	28	78	61.21		
10	*RTO/RTO*	31	17	67.64	100.51	<0.001
	rto/rto	13	79	13.57		
Pooled	*RTO/RTO*	186	246	319.47	30.99	<0.001
	rto/rto	189	393	304.73		
Heterogeneity					83.34	<0.001

Table S1. Individual and overall results from pollinator cage trials when wild-type (*RTO/RTO*) flowers are competed against *rto*-like (*rto/rto*) flowers of SWC F_2 mapping population siblings. Related to Table 1. Expected visits are calculated proportional to the number of open flowers across the three plants of a given phenotype within each trial.

Trial	Genotype	No. Open Flowers	Observed Visits	Expected Visits	G-value	*P*
1	*RTO/RTO*	36	5	10.05	2.06	0.15
	RTO/rto	50	19	13.95		
2	*RTO/RTO*	30	23	16.40	1.87	0.17
	RTO/rto	45	18	24.60		
3	*RTO/RTO*	29	64	96.67	11.76	0.00
	RTO/rto	19	96	63.33		
4	*RTO/RTO*	31	42	78.96	17.89	0.00
	RTO/rto	22	93	56.04		
5	*RTO/RTO*	45	51	88.01	18.59	0.00
	RTO/rto	23	82	44.99		
6	*RTO/RTO*	28	59	64.78	0.49	0.48
	RTO/rto	23	59	53.22		
7	*RTO/RTO*	18	62	50.31	2.18	0.14
	RTO/rto	21	47	58.69		
8	*RTO/RTO*	45	56	57.93	0.06	0.81
	RTO/rto	42	56	54.07		
9	*RTO/RTO*	46	46	46.95	0.02	0.90
	RTO/rto	51	53	52.05		
10	*RTO/RTO*	67	42	42.19	0.00	0.98
	RTO/rto	68	43	42.81		
Pooled	*RTO/RTO*	375	450	552.25	17.92	<0.001
	RTO/rto	364	566	463.75		
Heterogeneity					37.01	<0.001

Table S2. Individual and overall results from pollinator cage trials when wild-type (*RTO/RTO*) flowers are competed against intermediate phenotype (*RTO/rto*) flowers of SWC F_2 mapping population siblings. Related to Table 1. Expected visits are calculated proportional to the number of open flowers across the three plants of a given phenotype within each trial.

Trial	Genotype	No. Open Flowers	Observed Visits	Expected Visits	G-value	*P*
1	*RTO/rto*	30	4	10.88	3.34	0.07
	rto/rto	61	29	22.12		
2	*RTO/rto*	33	50	46.81	0.15	0.70
	rto/rto	53	72	75.19		
3	*RTO/rto*	23	48	37.33	1.91	0.17
	rto/rto	46	64	74.67		
4	*RTO/rto*	29	38	40.00	0.09	0.77
	rto/rto	29	42	40.00		
5	*RTO/rto*	10	23	20.80	0.17	0.68
	rto/rto	15	29	31.20		
6	*RTO/rto*	39	33	28.04	0.67	0.41
	rto/rto	50	31	35.96		
7	*RTO/rto*	21	52	58.03	0.60	0.44
	rto/rto	17	53	46.97		
8	*RTO/rto*	34	72	58.37	3.31	0.07
	rto/rto	26	31	44.63		
9	*RTO/rto*	25	43	31.77	3.72	0.05
	rto/rto	23	18	29.23		
10	*RTO/rto*	15	41	49.29	1.30	0.25
	rto/rto	13	51	42.71		
Pooled	*RTO/rto*	259	404	381.32	1.09	0.30
	rto/rto	333	420	442.68		
Heterogeneity					14.18	0.12

Table S3. Individual and overall results from pollinator cage trials when intermediate phenotype (*RTO/rto*) flowers are competed against *rto*-like (*rto/rto*) flowers of SWC F_2 mapping population siblings. Related to Table 1. Expected visits are calculated proportional to the number of open flowers across the three plants of a given phenotype within each trial.

Marker/Gene	Dir.	Primer Sequence (5' to 3')	Marker Type*	Position**
MgSTS92	F	CACGACGTTGTAAAACGACCAGCTCGTCGAACTTTGTCA	EPIC	17041346 - 17043324
	R	GTTTCTTTTGGTTCATCGATCTCCACA		
MgSTS513	F	CACGACGTTGTAAAACGACTTGACCATCATCTTTGACAAGC	EPIC	17267209 - 17274425
	R	GTTTCTTGAAGCAGGAGTCATCGAACC		
SWC_LG2.5	F	CACGACGTTGTAAAACGATGCCGGAAATAGCACACA	Micro-satellite	18599750
	R	GTTTCTTATAGAACCCATAATTGCCAACA		
SWC_LG2_ CAPS2	F	CCTGCAATGTTTCGTCACTAAC	CAPS (BspHI)	18676874
	R	CAACTACTACCACAGGAAGCAA		
Migut. B01849	F	GGGTGCAGAGGATAGAAGTAATG	Indel	18679492
	R	GCATAATCCAATCTTTGGGTACAAT		
SWC_LG2_ CAPS5	F	ATTGTTACCTGGAATAGCCTTCT	CAPS (HpaII)	18688069
	R	AGCCCTCTTCTTACAACATAGC		
SWC_LG2_ CAPS3	F	CCATTCTGGCTTCTATGGGATAC	CAPS (EcoRV)	18703614
	R	TACTCGATATGGCGGAGGAATA		
MgSTS249	F	CACGACGTTGTAAAACGATCTGATTTTTGCTGGGAAGC	EPIC	18963030 - 18964853
	R	GTTTCTTGCCAAAGCCATCAAAGAAGG		
MgSTS652	F	CACGACGTTGTAAAACGACTGCCATTGGTCCTCAACC	EPIC	18971779 - 18974369
	R	GTTTCTTAGCTTTTGACCATTTTGAGC		

Table S4. Primers used in *M. guttatus* fine mapping experiment. Related to Figure 2. *EPIC = exon-primed intron crossing (www.mimulusevolution.org); restriction enzyme used is provided for cleaved, amplified polymorphic sequence (CAPS) markers. **Position is provided in reference to chromosome 2 of the *Mimulus guttatus* v2.0 genome (www.phytozome.org); start and end positions of the amplified EPIC fragments are given since specific variants causing fragment length differences between cross parents were not identified by sequencing.

Primers	Sequence (5'-3')	Plasmid
*MlRTO*_RNAi_F	GTTCTAGACCATGGCAGCTTCACTCTCAGCTTTACT	*MlRTO_RNAi*
*MlRTO*_RNAi_R	GTGGATCCGGCGCGCCCAAGCTTGTGCATTCTGCAGA	*MlRTO_RNAi*
*MgRTO*_RNAi_F	GTTCTAGACCATGGATGGATAATAATAATAAAACTAG	*MgRTO_RNAi*
*MgRTO*_RNAi_R	GTGGATCCGGCGCGCCTTATTTTTTCCTAGTTGTTTGTGG	*MgRTO_RNAi*
*MgNEGAN*_RNAi_F	GTTCTAGACCATGGAAGCGATTACGTCCACCAACATCG	*MgNEGAN_RNAi*
*MgNEGAN*_RNAi_R	GTGGATCCGGCGCGCCTTAATTAGGCCCCAGTAGGCCCCACG	*MgNEGAN_RNAi*
*MlRTO*_cdsF	**cacc**ATGGATAATACTAAGCAAAATC	*35S:RTO,* *35S:YFP-RTO,* *pUBC:RTO-cYFP,* *pUBC:rto-cYFP*
*MlRTO*_cdsR	TCAAGAATTATCATGTTTCCTGGT	*35S:RTO,* *35S:YFP-RTO*
*MlRTO*_cdsR-NS	AGAATTATCATGTTTCCTGGTTTGT	*pUBC:RTO-cYFP*, *pUBC:rto-cYFP*
*MlANbHLH1*_cdsF	**cacc**ATGGCTGCTGGAAACCAAGACCAA	*pUBC:ANbHLH1-nYFP*
*MlANbHLH1*_cdsR	ACACTTTCTGATAACTTTCTGAAGAGC	*pUBC:ANbHLH1-nYFP*
*AatII_RTO*_ProF	GTGACGTCCTCGTGAAATTGGATAAGAAATCT	*RTO_{pro}:CFP-ER,* *RTO_{pro}:YFP-RTO*
*XhoI_RTO*_ProR	CCGCTCGAGCGGAGTAAAGCTGAGAGTGAAGCTGAT	*RTO_{pro}:CFP-ER,* *RTO_{pro}:YFP-RTO*
*CFP-ER*_cdsF	**cacc**ATGAAGGTACAGGAGGGTTTGT	*RTO_{pro}:CFP-ER*
*CFP-ER*_cdsR	TTACAGCTCGTCATGAGATCTCTT	*RTO_{pro}:CFP-ER*
sgRNA4_F	GTTCGAATGCACAAGCTCGTGTTTAAGAGCTATGCTGGAA	*MgRTO_CRISPR*
sgRNA4_R	ACGAGCTTGTGCATTCGAACAATCACTACTTCGTCTCTAAC	*MgRTO_CRISPR*
AtU6_F	CGAGGCGCGCCAGAAATCTCAAAATTCCG	*MgRTO_CRISPR*
AtU6_R	CGATTAATTAACATTTTACATAACAATAGTGA	*MgRTO_CRISPR*

Table S5. Primers used for plasmid construction. Related to Figures 3-5. The underlined sequences contain the restriction sites. The sequence highlighted in bold ("cacc") is the 4-bp sequence necessary for pENTR/D-TOPO cloning. *Ml*: *Mimulus lewisii*; *Mg*: *Mimulus guttatus*.

Gene	Dir.	Sequence (5'-3')
MlNEGAN	F	ATGGGATACTGGTCGCCGGCGAAGA
MlNEGAN	R	ATTWGGCCCCAGTAGGCCCCACGAA
MlRTO	F	ATGGATAATACTAAGCAAAATC
MlRTO	R	TCAAGAATTATCATGTTTCCTGGT
MlUBC	F	GGCTTGGACTCTGCAGTCTGT
MlUBC	R	TCTTCGGCATGGCAGCAAGTC
MgNEGAN	F	TTAGAGCAGGGCTGAACAGATG
MgNEGAN	R	TGTTCCAGACGTTCTTCACGTCG
MgRTO	F	AGCATGAGTGAGCAAGAAC
MgRTO	R	CAATCTCTTGTGCAGTCCTC
MgUBQ	F	GCGCAAGAAGAAGACGTACAC
MgUBQ	R	CTTCTTCAGCCTCTGCACCT
NbACTIN	F	CTGAGAGATTCCGCTGC
NbACTIN	R	GAGGACAATGTTTCCGTAC

Table S6. Primers used for RT-PCRs. Related to Figures 2 and 3. *Ml*: *Mimulus lewisii*; *Mg*: *Mimulus guttatus*; *Nb*: *Nicotiana benthamiana*.